Immunity
Article

Regulation of Immunoglobulin Light-Chain Recombination by the Transcription Factor IRF-4 and the Attenuation of Interleukin-7 Signaling

Kristen Johnson,[2,6] Tamar Hashimshony,[2,6] Catherine M. Sawai,[3,4] Jagan M.R. Pongubala,[1,2] Jane A. Skok,[4,5] Iannis Aifantis,[3,4] and Harinder Singh[1,2,*]
[1]Howard Hughes Medical Institute
[2]Department of Molecular Genetics and Cell Biology
[3]Section of Rheumatology, Department of Medicine
The University of Chicago, 929 East 57th Street, GCIS W522, Chicago, IL 60637, USA
[4]Department of Pathology, New York University School of Medicine, New York, NY 10016, USA
[5]Department of Immunology and Molecular Pathology, University College of London, London W1T 4JF, UK
[6]These authors contributed equally to this work.
*Correspondence: hsingh@uchicago.edu
DOI 10.1016/j.immuni.2007.12.019

SUMMARY

Productive rearrangement of the immunoglobulin heavy-chain locus triggers a major developmental checkpoint that promotes limited clonal expansion of pre-B cells, thereby culminating in cell-cycle arrest and rearrangement of light-chain loci. By using *Irf4*$^{-/-}$ *Irf8*$^{-/-}$ pre-B cells, we demonstrated that two pathways converge to synergistically drive light-chain rearrangement, but not simply as a consequence of cell-cycle exit. One pathway was directly dependent on transcription factor IRF-4, whose expression was elevated by pre-B cell receptor signaling. IRF-4 targeted the immunoglobulin 3′Eκ and Eλ enhancers and positioned a kappa allele away from pericentromeric heterochromatin. The other pathway was triggered by attenuation of IL-7 signaling and activated the iEκ enhancer via binding of the transcription factor E2A. IRF-4 also regulated expression of chemokine receptor *Cxcr4* and promoted migration of pre-B cells in response to the chemokine ligand CXCL12. We propose that IRF-4 coordinates the two pathways regulating light-chain recombination by positioning pre-B cells away from IL-7-expressing stromal cells.

INTRODUCTION

Multiple *cis*-acting elements and *trans*-acting factors control the developmentally regulated accessibility of Ig gene segments to the recombinase machinery (Schlissel, 2004). In differentiating pro-B cells, productive rearrangement of the Ig heavy-chain locus leads to the assembly of the pre-B cell receptor (pre-BCR) (Geier and Schlissel, 2006). Signaling through the pre-BCR and the IL-7 receptor (IL-7R) drives proliferation and enables clonal expansion of pre-B cells. Intriguingly, pre-BCR expression reduces the dependence of developing B cells on IL-7 signaling (Milne and Paige, 2006; Rolink et al., 2000). Consistent with these findings, pre-B cells are positioned away from IL-7-expressing stromal cells in the bone marrow (Tokoyoda et al., 2004). Upon cessation of proliferation, cells transit into the small pre-B cell stage and induce Ig light-chain recombination. Despite considerable work, the integration of these signaling pathways and their coupling to nuclear regulators of Ig light-chain recombination remain poorly understood.

Contrasting arguments have been advanced concerning the requirement for acquired pre-BCR or attenuated IL-7 signaling in activating Ig light-chain recombination. Signaling by the pre-BCR is widely considered to promote light-chain recombination. As such, enforced expression of a rearranged *Igh* transgene increases *Igk* locus accessibility in RAG-deficient pro-B cells (Stanhope-Baker et al., 1996). Additionally, expression of a constitutively active Ras protein, a signaling molecule downstream of the pre-BCR, promotes light-chain recombination in the absence of a rearranged heavy-chain (Shaw et al., 1999), whereas loss of downstream components of the pre-BCR signaling cascade, including BLNK, Btk, and PLCγ, results in fewer cells that are able to rearrange their kappa loci (Flemming et al., 2003; Xu et al., 2007). However, pro-B cells can undergo *Igk* rearrangement in the absence of a pre-BCR (Grawunder et al., 1993). Therefore, the developmental requirement of the pre-BCR and the molecular pathway by which it promotes light-chain recombination remain unresolved. Similarly, the role of attenuated IL-7 signaling in the promotion of light-chain recombination is unclear. Although withdrawal from IL-7 leads to increased *Igk* recombination in vitro, *Igk* rearrangement can be detected in pre-BCR^{+} cells that are cultured in a high concentration of IL-7 (Rolink et al., 2000). It has been argued that IL-7 withdrawal merely leads to the selective survival of IgM^{+} cells that have undergone productive light-chain rearrangement (Milne et al., 2004). Paradoxically, the only established role for IL-7 signaling in controlling recombination of immunoglobulin loci involves positive regulation of distal V_H gene accessibility via the transcription factor Stat5 (Bertolino et al., 2005). Thus, it remains to be established whether IL-7 signaling negatively regulates Ig light-chain locus accessibility, and if so, then the underlying mechanism is of considerable interest.

The *Igk* locus contains two distinct transcriptional enhancers, the intronic enhancer (iEκ) and the 3′ enhancer (3′Eκ) that function to regulate V(D)J recombination. *Igk* recombination is diminished upon deletion of either enhancer and completely abolished in the compound-mutant mice (Inlay et al., 2002). The transcription factors E2A and Pax5 are required for *Igk* rearrangement (Lazorchak et al., 2006; Sato et al., 2004). However, each of these factors also regulates heavy-chain rearrangement at the pro-B cell stage. Therefore, it is unclear how these factors selectively promote *Igk* rearrangement at the pre-B cell stage. In contrast, the related interferon regulatory factor (IRF) family members IRF-4 and IRF-8 have been demonstrated to be uniquely required for Ig light-chain recombination. *Irf4,Irf8* compound-mutant mice accumulate in their bone marrow cycling pre-B cells that fail to undergo light-chain recombination (Lu et al., 2003). Although IRF-4 and IRF-8 act in a redundant manner at the pre-B cell stage, IRF-4 functions exclusively later in B cell development to regulate Ig class-switch recombination and plasma cell differentiation (Klein et al., 2006; Sciammas et al., 2006). Intriguingly, in both pre-B and B cells, IRF-4 seems to function to limit clonal expansion and promote differentiation processes that involve recombination and expression of Ig genes (Lu et al., 2003; Sciammas et al., 2006). In both developmental stages, *Irf4* expression is induced downstream of the antigen receptor (Matsuyama et al., 1995; Muljo and Schlissel, 2003). We therefore considered the possibility that upregulation of IRF-4 expression at the pre-B cell stage could be used to trigger Ig light-chain recombination.

We have utilized $Irf4^{-/-}Irf8^{-/-}$ pre-B cells, which represent a unique experimental system, to analyze the roles of the pre-BCR and IL-7 signaling pathways in the activation of light-chain recombination. We demonstrate the existence of two distinct molecular pathways that function synergistically to promote Ig light-chain rearrangement. One pathway was strictly dependent on IRF-4, which targets the 3′Eκ and Eλ enhancers. The other pathway was triggered by attenuation of IL-7 signaling and resulted in activation of the iEκ enhancer via binding of the transcription factor E2A. Intriguingly, IRF-4 regulated the expression of *Cxcr4* and promoted the migration of pre-B cells in response to the chemokine CXCL12. IRF-4 can therefore position pre-B cells away from IL-7-expressing stromal cells. We propose that IRF-4 regulates Ig light-chain recombination by coordinating two molecular pathways that are triggered by acquired pre-BCR and attenuated IL-7 signaling, respectively.

RESULTS

Igk Recombination Can Be Induced via Two Independent Pathways in $Irf4^{-/-}Irf8^{-/-}$ Pre-B Cells

$Irf4^{-/-}Irf8^{-/-}$ pre-B cells can be propagated in culture on OP9 stromal cells with the cytokine IL-7 (Lu et al., 2003). The mutant pre-B cells express both the pre-BCR and the IL-7 receptor and therefore provide a unique experimental system for analyzing the role of each signaling pathway in regulating the induction of Ig light-chain gene rearrangements. By using an IRF-4 antibody, we noted that IRF-4 protein is elevated in pre-B cells (Figure S1 available online), consistent with elevated *Irf4* transcripts seen at this developmental stage (Muljo and Schlissel, 2003). Increased IRF-4 expression appears to require signaling through the pre-BCR because it is dependent on the adaptor protein SLP-65 (Thompson et al., 2007). Therefore, restoring expression of IRF-4 in the mutant cells should enable their pre-BCR-dependent differentiation. $Irf4^{-/-}Irf8^{-/-}$ pre-B cells were transduced with either a control or an IRF-4 expressing retrovirus. After 3 days, GFP^+ cells were isolated and analyzed for *Igk* locus recombination. We noted that IRF-4 was expressed in these cells at levels similar to that observed in wild-type pre-B cells (Figure S1). Importantly, IRF-4 induced $V\kappa$ recombination to all four functional $J\kappa$ gene segments (Figure 1A).

To determine whether diminished IL-7 signaling can independently induce *Igk* recombination, we cultured $Irf4^{-/-}Irf8^{-/-}$ pre-B cells in media containing varying concentrations of IL-7 (data not shown). Lowering the IL-7 concentration to 0.1 ng/ml ($IL\text{-}7^{lo}$) resulted in loss of phosphorylated Stat5, a component of IL-7 signaling, and impaired expression of the *Cish* gene, a Stat5 target (Figure S2). Surprisingly, we also detected efficient recombination of $V\kappa$ gene segments to all four $J\kappa$ segments upon lowering IL-7 concentration (Figure 1A). Importantly, *Igk* recombination was inducible within 48 hr of attenuating IL-7 signaling under conditions of minimal cell death (Figure 1B and data not shown). This suggests that it is a direct consequence of this perturbation and does not represent selective survival of B lineage cells that have undergone productive *Igk* rearrangement. We note that IRF-4-induced *Igk* light-chain recombination occurs in the presence of a high concentration of IL-7 (5 ng/ml), thereby demonstrating that IRF-4 can promote *Igk* recombination independently of attenuated IL-7 signaling (Figure 1A). Collectively, these data suggest that two distinct pathways can induce *Igk* light-chain recombination in pre-B cells: one that is strictly dependent on IRF-4, a pre-BCR inducible transcription factor, and the other on attenuated IL-7 signaling.

IRF-4 Expression and Attenuation of IL-7 Signaling Function Synergistically to Promote *Igk* Recombination and IgM Expression

To determine whether these pathways can function synergistically, $Irf4^{-/-}Irf8^{-/-}$ pre-B cells transduced with the control or the IRF-4 retrovirus were cultured in high or low concentrations of IL-7. When both pathways were engaged, there was a greater than additive increase in the frequency of *Igk* recombination (Figure 1B). After productive recombination, transcription of the rearranged light-chain gene results in the expression of a protein that pairs with the heavy-chain and is expressed on the cell surface. As expected, expression of IRF-4 in $Irf4^{-/-}Irf8^{-/-}$ pre-B cells induced the generation of IgM^+ B cells in the presence of high IL-7 (1.8%, Figure 1C). Intriguingly, upon the attenuation of IL-7 signaling, despite similar amounts of *Igk* DNA recombination, $Irf4^{-/-}Irf8^{-/-}$ cells did not give rise to IgM^+ B cells. However, the combination of IRF-4 re-expression and attenuation of IL-7 signaling resulted in a large increase in the frequency of generation of IgM^+ B cells (7.7%, Figure 1C). Therefore, IRF-4 expression and attenuated IL-7 signaling function synergistically not only to induce *Igk* light-chain recombination but also to promote the generation of IgM^+ B cells.

Cell-Cycle Exit Is Not Sufficient to Induce *Igk* Recombination in Pre-B Cells

Upon lowering of IL-7 concentration, $Irf4^{-/-}Irf8^{-/-}$ pre-B cells stop proliferating as indicated by the decrease in forward scatter

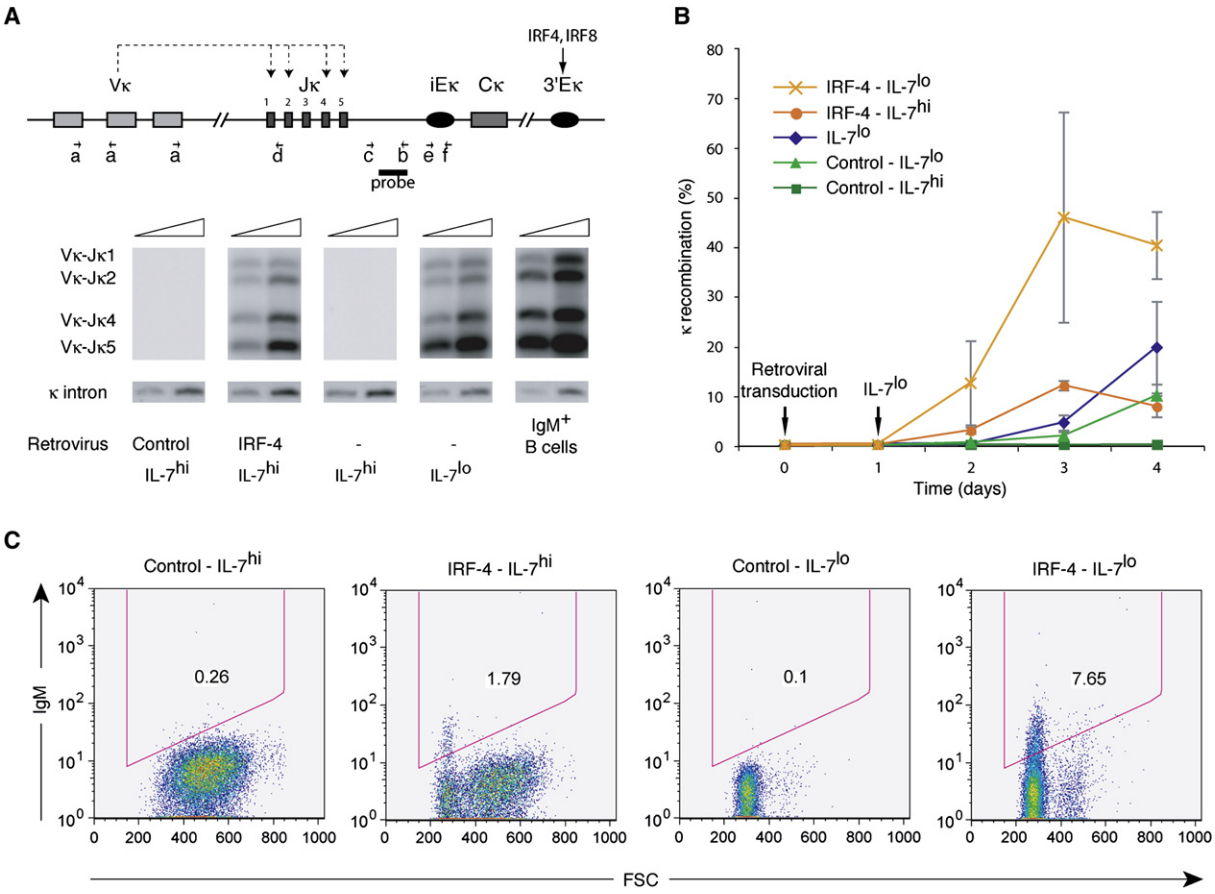

Figure 1. *Igk* Recombination Is Induced in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ Pre-B Cells via Two Independent Pathways

(A) Semiquantitative PCR analysis of *Igk* rearrangements in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells transduced with control (MigR1) or IRF-4 retroviral vectors. GFP-sorted cells were cultured in 5 ng/ml of IL-7 (IL-7^{hi}) for 3 days. IL-7 signaling was attenuated in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells by culturing in 0.1 ng/ml of IL-7 (IL-7^{lo}) for 3 days. DNA from splenic IgM^{+} B cells was used as the positive control. PCR reactions employed a degenerate *Vκ* primer and *Igk* intron primer (primers "a" and "b"). We amplified a region upstream of the *Igk* intron to control for amount of genomic DNA (primers "c" and "b"). Amplified products with 3-fold template dilutions were detected by hybridization with an *Igk* intron probe. Data are representative of five independent experiments that utilized three independently derived *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cell lines. A schematic of the *Igk* locus including primers and probes is depicted (not to scale).

(B) *Igk* rearrangements involving *Jκ1* were assayed under the indicated conditions by Q-PCR and measured relative to recombination frequency in IgM+ splenic B cells. We used amplification of iEκ to control for amount of DNA.

(C) Generation of surface IgM^{+} B cells from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells. The mutant cells were transduced with control or IRF-4 retrovirus and then cultured in either IL-7^{hi} or IL-7^{lo} for two additional days. FACS plots of IgM versus forward scatter are shown after gating on live GFP^{+} cells. All experiments were performed at least twice, and representative ones are shown. Error bars in (B) indicate the standard deviation.

by FACS (Figure 1C). Interestingly, a similar decrease is observed in a subset of IRF-4-transduced cells, and these are the cells that become IgM positive. To directly analyze cell-cycle status upon IRF-4 expression or attenuation of IL-7 signaling, we stained cells with the DNA-intercalating dye, Hoechst. IRF-4 expression led to an increase in the percentage of cells in G0/G1 (Figure 2A, top panel). Attenuation of IL-7 signaling resulted in a more pronounced accumulation of cells in G0/G1 (Figure 2A, bottom panel). These observations raised the possibility that both pathways induce light-chain recombination in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells as a consequence of triggering cell-cycle arrest. This hypothesis predicted that inhibiting proliferation of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells, via perturbation of cell-cycle regulators, should result in the induction of light-chain recombination. We tested this hypothesis in vivo as well as in vitro. The proliferation of wild-type pre-B cells is primarily dependent on cyclin D3 (Cooper et al., 2006). As expected, *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells proliferating in the presence of IL-7 expressed high amounts of cyclin D3 and low amounts of the cell-cycle inhibitor p27 (Figure 2B). Upon attenuation of IL-7 signaling, cyclin D3 amounts are strongly reduced, whereas p27 amounts increase, and the cells arrest in G0/G1. To genetically test the forementioned hypothesis, we generated *Irf4*$^{-/-}$*Irf8*$^{-/-}$*Ccnd3*$^{-/-}$ compound-mutant mice. Despite the fact that the *Irf4*$^{-/-}$*Irf8*$^{-/-}$*Ccnd3*$^{-/-}$ mice had a higher proportion of pre-B cells in G0/G1, we detected no *Igk* light-chain recombination or a rescue of the B cell developmental defect (Figure 2C and data not shown). We also tested whether enforced expression of p27 in cycling *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells would induce light-chain recombination. *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells were transduced with either the control or the p27 retrovirus, sorted 2 days later, and analyzed for *Igk* recombination. The p27-transduced cells accumulated in G0/G1 but did not induce

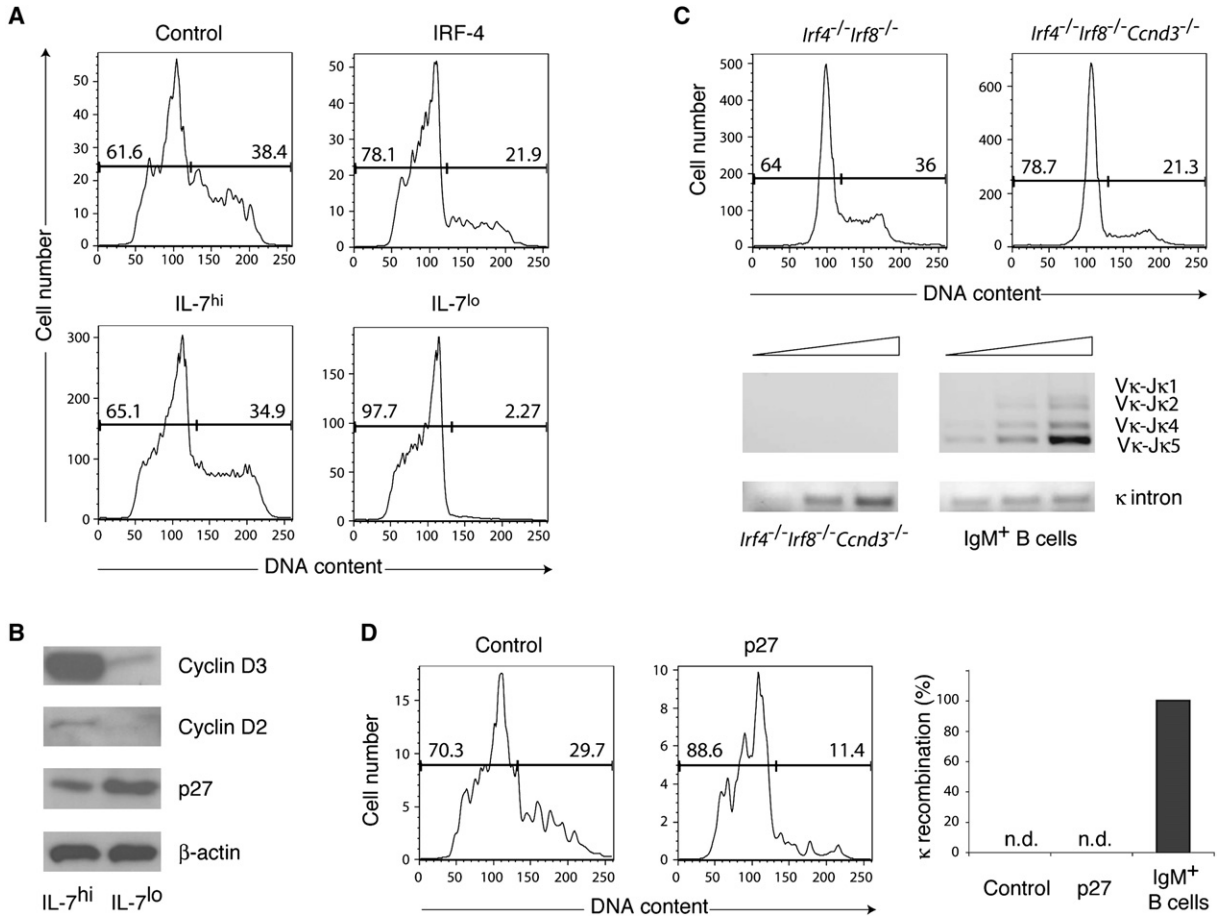

Figure 2. Cell-Cycle Arrest Is Not Sufficient to Induce *Igk* Rearrangement in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ Pre-B Cells

(A) Cell-cycle analysis of Hoechst-stained *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells under the indicated conditions. Transduced cells were analyzed 3 days after culture in IL-7^{hi} conditions. Samples were gated for GFP expression. IL-7^{lo} cells were assayed 1 day after transfer from IL-7^{hi} conditions.

(B) Immunoblot analysis of cell-cycle regulators in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells cultured in IL-7^{hi} or IL-7^{lo} for 2 days. Protein expression was normalized to β-actin.

(C) Cell-cycle analysis of B220^{+}, CD43^{-}, and CD25^{int} pre-B cells from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ or *Irf4*$^{-/-}$*Irf8*$^{-/-}$*Ccnd3*$^{-/-}$ mice. Semiquantitative PCR analysis of *Igk* recombination (as described in Figure 1A) in pre-B cells from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ mice is shown.

(D) Cell-cycle analysis of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells after 1 day subsequent to transduction with control or p27 vector. *Igk* recombination was assayed 2 days after transduction by Q-PCR as described in Figure 1B ("n.d." stands for not detectable). Data are representative of at least two independent experiments.

rearrangement of their *Igk* light-chain loci (Figure 2D). Thus, cell-cycle arrest is not a sufficient developmental trigger for inducing Ig light-chain recombination in pre-B cells.

IRF-4 Expression and Attenuation of IL-7 Signaling Differentially Induce Germline *Igk* and *Rag1* transcripts

In order to molecularly analyze the means by which the two pathways induce Ig light-chain recombination, we initially focused on the activation of *Igk* germline and *Rag* gene transcription. The former is tightly correlated with the potential to undergo recombination and is considered to reflect a *Igk* locus that is accessible to the recombinase machinery (Schlissel, 2004). *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells have a profound block in *Igk* germline transcription (Lu et al., 2003). Restoration of IRF-4 expression in the mutant pre-B cells induced a robust increase in *Igk* germline transcription to a degree similar to that seen in wild-type small pre-B cells (Figure 3A and Figure S3A). Attenuation of IL-7 signaling also resulted in activation of *Igk* germline transcription albeit at amounts 5%–10% of those observed upon IRF-4 expression (Figure 3A). Thus, although both pathways modulate accessibility of the *Igk* locus, IRF-4 is a more potent inducer of *Igk* germline transcription.

Irf4$^{-/-}$*Irf8*$^{-/-}$ pre-B cells are also impaired for the expression of the *Rag* genes. In contrast to the pattern observed for *Igk* germline transcription, *Rag1* transcription was modestly induced by IRF-4 (3- to 4-fold) expression but highly induced upon attenuation of IL-7 signaling (~100-fold) (Figure 3B), although this amount of induction is somewhat lower than found in wild-type small pre-B cells (Figure S3B). A similar pattern was observed with the *Rag2* gene, although the induction ratios were lower (data not shown). It is important to note that the robust induction of *Rag1* transcription upon attenuation of IL-7 signaling is not simply a consequence of cell-cycle exit because the IRF-4- or p27-transduced cells exhibit modest increases in *Rag1* transcription (Figure 3B and data not shown).

To determine whether engagement of both pathways resulted in synergistic increases in *Igk* germline or *Rag1* transcription, we analyzed cells that had been transduced with either control or

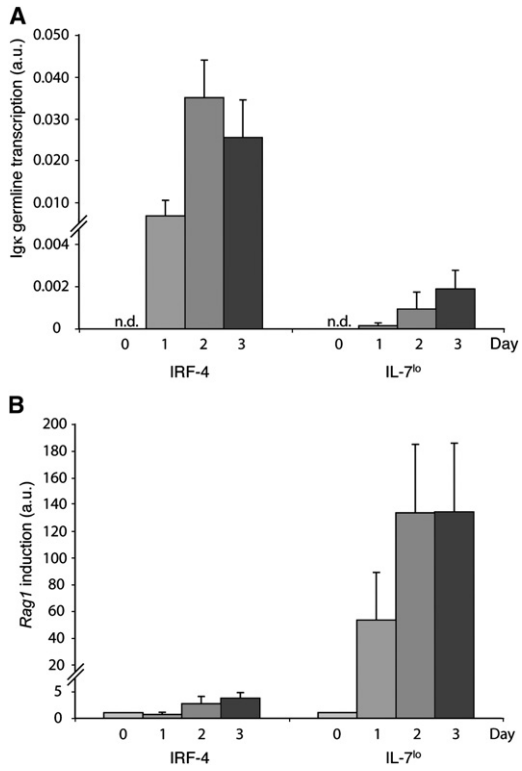

Figure 3. IRF-4 Expression and Attenuation of IL-7 Signaling Differentially Induce *Igk* Germline and *Rag1* Transcription

Indicated RNA transcripts were analyzed by Q-PCR relative to β_2-microglobulin in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells after transduction with IRF-4 retrovirus or shifting to IL-7^{lo} conditions (1–3 days). As shown in (A), quantitation of *Igk* germline transcripts initiated from the J_κ proximal promoter. As shown in (B), *Rag1* transcripts are represented as fold induction either upon lowering IL-7 concentration (IL-7^{lo} relative to IL-7^{hi}) or upon IRF-4 transduction (IRF-4 relative to control). "a.u." denotes arbitrary units. Data represent an average of six experiments. Error bars indicate standard deviation.

IRF-4 retrovirus and shifted to the low concentration of IL-7. Interestingly, we did not observe a synergistic effect on *Igk* germline or *Rag1* transcription (data not shown). Thus, the IRF-4 pathway preferentially induces *Igk* germline transcription, whereas modulating IL-7 signaling more potently activates *Rag* gene expression. Therefore, the synergistic increase in *Igk* recombination as a consequence of engaging both pathways (Figure 1B) is partly attributable to their differential effects on locus accessibility and *Rag* gene expression (see below).

IRF-4 Counteracts Association of an *Igk* Allele with Pericentromeric Heterochromatin

In pro-B cells, both *Igk* alleles are repositioned away from the nuclear periphery, and such relocation has been suggested to promote accessibility to recombination (Kosak et al., 2002). However, in pre-B cells, one of the *Igk* alleles becomes associated with pericentromeric heterochromatin (Goldmit et al., 2005), a process that has been suggested to favor rearrangement of the allele that is positioned away from pericentromeric heterochromatin. We used 3D fluorescence in situ hybridization to analyze the positioning of germline *Igk* alleles in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells. Intriguingly, these cells displayed a high proportion of nuclei in which both *Igk* alleles were associated with pericentromeric heterochromatin (Figure 4). This is in contrast to CD19^{+}IgM^{-} wild-type B lineage cells or sorted pre-B cells in which only a small proportion of nuclei displayed biallelic association (Figure 4 and Goldmit et al. [2005]). The atypical association of *Igk* alleles with heterochromatin in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells did not change upon lowering IL-7 signaling. These data raised the possibility that IRF-4 participates in positioning one *Igk* allele away from pericentromeric heterochromatin. We therefore analyzed the nuclear configuration of *Igk* alleles in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells after restoration of IRF-4 expression. Importantly, in IRF-4-transduced cells, a low proportion of nuclei displayed biallelic association, and thus their nuclear distribution of *Igk* alleles was similar to that of wild-type cells (Figure 4). These data demonstrate that IRF-4 functions in positioning an *Igk* allele away from pericentromeric heterochromatin.

Recombination of the *Igλ* Locus Is Dependent on IRF-4

Irf4$^{-/-}$*Irf8*$^{-/-}$ pre-B cells are also deficient in *Igλ* germline transcription and recombination (Lu et al., 2003). Therefore, we sought to determine the effects of both pathways on *Igλ* germline transcription and recombination. Strikingly, whereas restoration of IRF-4 expression induced both *Igλ* germline and rearranged *Igλ* transcripts, attenuation of IL-7 signaling did not appreciably activate these processes (Figure S4). Therefore, IRF-4 is required for the activation of Igλ germline transcription as well as recombination.

IRF-4 Expression and Attenuation of IL-7 Signaling Induce Histone H4 Hyperacetylation at Distinct Ig Light-Chain Enhancers

One major difference between *Igλ* and *Igk* loci is their enhancers. The *Igλ* locus has two duplicated enhancers, each of which has a functional Ets-IRF composite binding site for IRF-4,8 (Eisenbeis et al., 1995). In contrast, the *Igk* locus has two distinct enhancers, the intronic enhancer (iEκ) and the 3′ enhancer (3′Eκ); only the latter contains a functional Ets-IRF composite binding site for IRF-4,8 (Pongubala et al., 1992). Because Igλ recombination is highly dependent on IRF-4, we hypothesized that IRF-4 promotes recombination of *Igk* and *Igλ* loci via direct engagement of the 3′Eκ and Eλ enhancers, respectively. In contrast, we reasoned that attenuated IL-7 signaling may selectively induce *Igk* recombination via activation of the iEκ enhancer. In support of this idea, the J_κ usage observed in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells upon inducing recombination by lowering IL-7 signaling (Figure S5) mimics the usage in 3′Eκ null cells (Inlay et al., 2002). To test these hypotheses, we assessed the activity of the endogenous enhancers in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells by analyzing their chromatin status. Histone-H4 acetylation levels were measured by chromatin immunoprecipitation (ChIP). The EL4 T cell line and *Rag1*$^{-/-}$ pro-B cell line were used as controls. The germline *Igk* and Igλ alleles in EL4 cells were transcriptionally inactive (data not shown) and exhibited background histone H4 acetylation (Figure S6). In contrast, pro-B cells had a substantial degree of H4 acetylation associated with the light-chain enhancers. Consistent with the hypothesis that IRF-4,8 regulate the activities of the 3′Eκ and Eλ$_{1\text{-}3}$ enhancers, the degree of histone acetylation at these enhancers was severely compromised in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells (Figure S6). We next determined

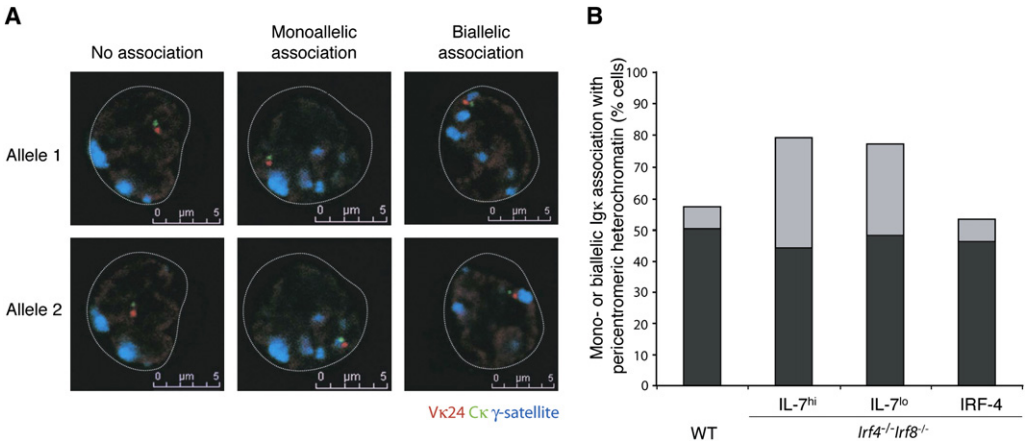

Figure 4. IRF-4 Promotes Positioning of One *Igk* Allele Away from Pericentromeric Heterochromatin

(A) Three-dimensional FISH analysis of *Igk* alleles in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells with probes to *Vκ24* (red), *Cκ* (green), and γ-satellite (blue). Both alleles were scored for association with pericentromeric heterochromatin as determined by colocalization of the *Igk* and γ-satellite signals. An example of each configuration is shown. Each allele within a single nucleus is shown in a distinct confocal section.

(B) Quantitative analysis of *Igk* locus association with pericentromeric heterochromatin under the indicated conditions: *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells cultured in IL-7^{hi} and IL-7^{lo} (1 day) conditions or transduced with IRF-4 retrovirus (2 days) after culture in IL-7^{hi}. *Igk* nuclear configurations are compared to those in CD19^{+}IgM^{-} cultured in IL-7^{lo}. Greater than 70 nuclei were scored for each condition. Black- and gray-shaded regions denote monoallelic and biallelic association, respectively.

whether IRF-4 expression could restore histone acetylation at 3′Eκ and Eλ$_{1\text{-}3}$. Histone H4 acetylation increased in response to IRF-4 at the 3′Eκ and Eλ$_{1\text{-}3}$ enhancers (~4-fold and ~2.5-fold, respectively; Figure 5A). Importantly, no change in histone acetylation was seen at iEκ under these conditions, demonstrating that IRF-4 selectively activates 3′Eκ and Eλ$_{1\text{-}3}$.

To test whether lowering of IL-7 signaling preferentially induces the activity of iEκ, we compared the degree of H4 acetylation at *Igk* and *Igλ* enhancers in the presence of either high or low concentrations of IL-7. As predicted by our hypothesis, we observed a ~4-fold increase in histone H4 acetylation at iEκ, upon attenuation of IL-7 signaling (Figure 5B). These data suggest that IL-7 signaling negatively regulates *Igk* recombination via repression of iEκ activity

Attenuation of IL-7 Signaling Enables E2A Binding at the *Igk* Intronic Enhancer

The transcription factor E2A is required for light-chain recombination and its protein expression has been shown to increase at the pre-B cell stage (Quong et al., 2004). E2A is required for iEκ activity because a compound mutation of its binding sites in iEκ is functionally equivalent to deletion of the entire enhancer (Inlay et al., 2004). Therefore, we tested the possibility that IL-7 signaling was negatively regulating *Igk* recombination via iEκ by modulating E2A protein expression or binding activity. Neither E2A protein expression nor DNA binding activity was affected by attenuating IL-7 signaling in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells (Figure S7). We next tested whether E2A occupancy of its binding sites in iEκ was negatively regulated by IL-7 signaling by performing ChIP assays. Strikingly, we observed that although E2A binding remains constant at the heavy-chain intronic enhancer (Eμ), it increased at iEκ upon attenuation of IL-7 signaling (Figure 5C). Consistent with data that E2A binding at 3′Eκ requires IRF-4 (Lazorchak et al., 2006), we observed no change in E2A occupancy at the 3′Eκ enhancer. Collectively, these data suggest that IL-7 signaling negatively regulates *Igk* recombination by antagonizing E2A binding at the iEκ enhancer.

IRF-4 Regulates *Cxcr4* Expression and Promotes Pre-B Cell Migration in Response to CXCL12

Our results raised a developmental conundrum. In vivo, light-chain recombination is completely dependent on IRF-4,8 (Lu et al., 2003), whereas in vitro, only one of the two pathways delineated above strictly requires IRF-4. We considered two mutually nonexclusive possibilities to explain this paradox: (1) IRF-4 upon its induction as a consequence of pre-BCR signaling antagonizes signaling through the IL-7 receptor, and (2) IRF-4 regulates the expression of chemokine receptors and/or adhesion molecules that can reposition pre-B cells away from stromal cells expressing IL-7, thereby attenuating IL-7 signaling. To explore both possibilities, we performed genome-wide expression analysis with *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells after IRF-4 transduction or attenuation of IL-7 signaling. This analysis revealed a large set of genes that were regulated by IL-7 signaling independently of IRF-4 (Figure S8). Therefore, IRF-4 appears not to antagonize IL-7 signaling. Consistent with our previous data (Figure 3), *Igk* transcripts were more highly induced by IRF-4, whereas attenuation of IL-7 signaling strongly upregulated *Rag1* and *Rag2* transcripts (Table S1). Interestingly, transcripts for DNA ligase IV, the enzyme that joins DNA ends during the process of V(D)J recombination, were also strongly induced by attenuation of IL-7 signaling (Table S1).

Intriguingly, IRF-4 induced the expression of a number of genes encoding chemokine receptors and adhesion molecules (Table S1). Of particular interest was the upregulation of *Cxcr4*, the receptor for CXCL12, and this result was confirmed by Q-PCR (data not shown). CXCL12 is expressed by a distinct set of bone marrow stromal cells that are spatially separated from IL-7-expressing stromal cells (Tokoyoda et al., 2004). Because pre-B cells are not found to be associated with

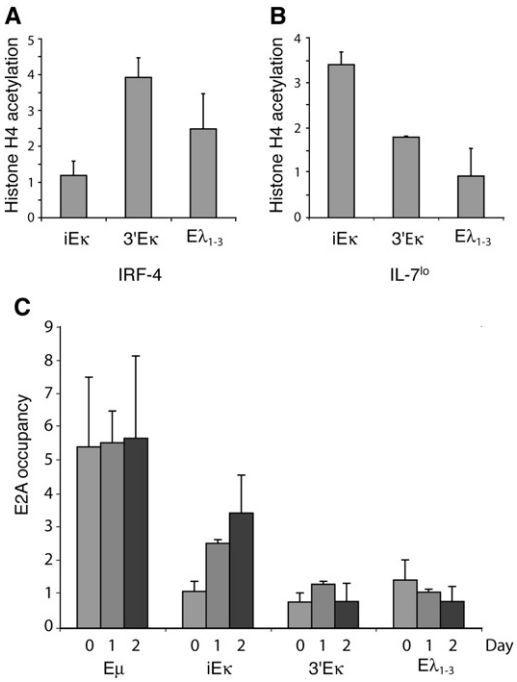

Figure 5. IRF-4 Expression and Attenuation of IL-7 Signaling Differentially Induce Histone Acetylation and E2A Binding at Ig Light-Chain Enhancers

Chromatin crosslinking and immunoprecipitation assays (ChIPs) with acetylated histone H4 or E2A antibodies in the indicated cell types. Relative enrichment of the bound DNA over input was determined by Q-PCR after normalization to α-actin. (A) shows the fold change in H4 acetylation levels at light-chain enhancers in sorted *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells 2 days after IRF-4 transduction (IRF-4 relative to control). (B) shows the fold change in H4 acetylation levels at light-chain enhancers upon lowering IL-7 for 1 day (IL-7^{lo} relative to IL-7^{hi}). As shown in (C), binding of E2A at light-chain enhancers was assessed in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells cultured in IL-7^{hi} (light-gray bar) or in IL-7^{lo} conditions for 1 (dark-gray bar) or 2 days (black bar). E2A binding to the Eμ heavy-chain enhancer was used as a positive control. Data are from three experiments. Error bars indicate standard deviation.

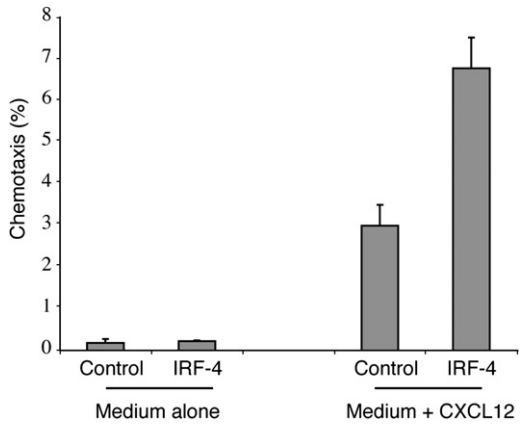

Figure 6. IRF-4 Promotes Migration of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ Pre-B Cells in Response to CXCL12

Migration behavior of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells transduced with a control or IRF-4 retrovirus in response to medium or 100 ng/ml of CXCL12, 2 days after transduction. Each transwell assay was performed in duplicate. The average percentage of input cells that migrated is shown from three independent experiments. Error bars indicate standard deviation.

IL-7-expressingstroma and display increased chemotaxis to CXCL12 (Tokoyoda et al., 2004), migration of pre-B cells toward a localized source of CXCL12 may provide a mechanism by which these cells can move away from IL-7 stroma, resulting in attenuation of IL-7 signaling. We therefore tested, by using transwell assays, whether IRF-4 promoted migration of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells to CXCL12. IRF-4-expressing cells showed an ~2.5-fold increase in migration in response to CXCL12 (Figure 6). These data suggest that IRF-4 regulates the migration of pre-B cells in the bone marrow, resulting in their movement away from IL-7-expressing stromal cells. This migration would lead to the attenuation of IL-7 signaling, thereby enabling the activation of both pathways that synergistically activate light-chain recombination (Figure 7).

DISCUSSION

Signaling by the pre-BCR and loss of IL-7 signaling have each been proposed to regulate light-chain recombination (Geier and Schlissel, 2006; Grawunder et al., 1993; Milne et al., 2004; Rolink et al., 2000). However, the evidence with different experimental systems has led to models in which the developmental activation of light-chain recombination is viewed as either pre-BCR or IL-7 dependent. Although these data could be interpreted as contradictory, another possibility is that parallel or synergistic pathways promote light-chain recombination such that interruption of either pathway still permits some level of recombination. In this regard, the *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cell phenotype is unique because there is a complete block to light-chain recombination (Lu et al., 2003). Because these cells express high levels of the pre-BCR and are strictly dependent on IL-7 for growth in vitro, they provide a distinctive and powerful model system for assessing the role of these two signaling pathways in light-chain recombination. By using nontransformed *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells, we unequivocally demonstrate that *Igk* light-chain recombination can either be directly induced by IRF-4, a regulatory factor whose expression is induced by signaling through the pre-BCR (Muljo and Schlissel, 2003; Thompson et al., 2007), or by modulation of IL-7 signaling. The two pathways are shown to differentially regulate the chromatin accessibility of light-chain loci and the expression of the recombinase genes. IRF-4 appears to coordinate both pathways by regulating migration of pre-B cells away from stromal cells expressing IL-7, thereby enabling synergistic induction of light-chain recombination.

Recently, transplantation experiments with *Irf4*$^{-/-}$*Irf8*$^{-/-}$ hematopoietic stem cells have been used to confirm that IRF-4 and IRF-8 function in a cell-autonomous manner to regulate pre-B cell differentiation (Ma et al., 2006). Importantly, the block in B cell development is not due to a defect in cell survival because it cannot be rescued by enforced expression of a *Bcl2* transgene. Additionally, restoration of either IRF-4 or IRF-8 expression in *Irf4*$^{-/-}$*Irf8*$^{-/-}$ pre-B cells could induce the generation of IgM expressing cells. However, these authors restored IRF-4 or 8 expression under conditions involving acute IL-7 withdrawal and therefore could not distinguish or molecularly analyze the contributions of the two pathways on light-chain recombination.

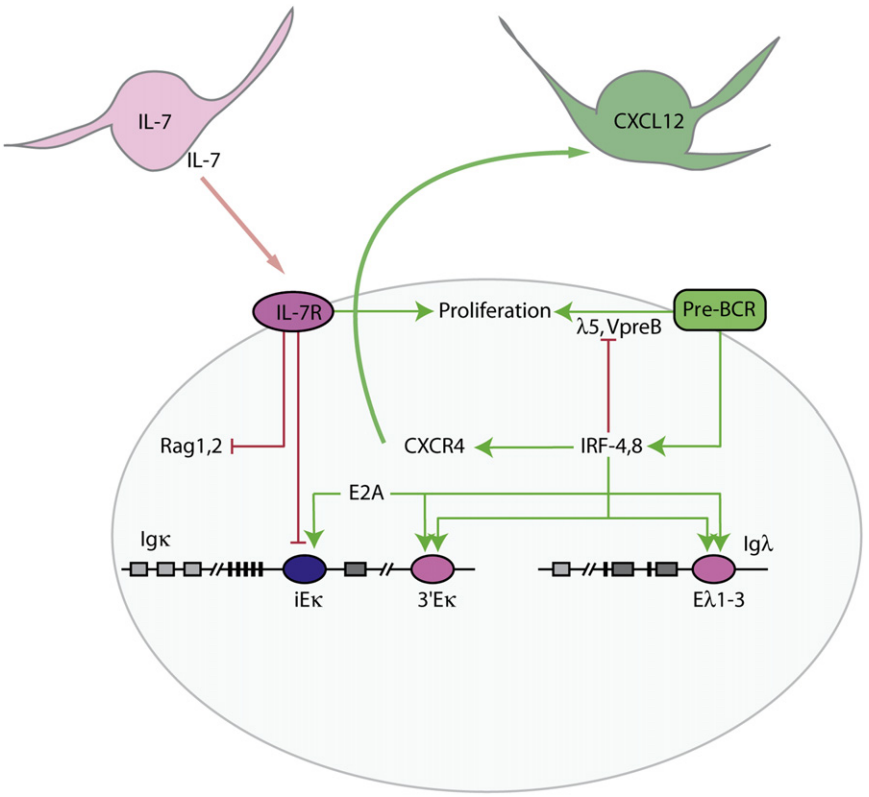

Figure 7. Integration of Pre-BCR and IL-7R Signaling Pathways via IRF-4 and the Regulation of Ig Light-Chain Rearrangement in Pre-B Cells

The regulatory network depicts signaling pathways and transcriptional regulators that are required for light-chain recombination at the pre-B cell stage. Arrows represent positive regulation, and barred lines represent repression. IRF-4 plays a central role in inducing light-chain recombination downstream of the pre-BCR by directly engaging the 3′Eκ and Igλ light-chain enhancers. IRF-4 is proposed to attenuate IL-7 signaling by moving pre-B cells away from IL-7-expressing stroma as a consequence of upregulation of the chemokine receptor *Cxcr4*. This results in robust induction of Rag gene expression, E2A binding to iEκ, and synergistic activation of light-chain recombination by the two pathways.

Our experimental design has enabled an unequivocal demonstration of the two pathways (pre-BCR and IL-7) that can function independently as well as synergistically to regulate light-chain recombination.

The two convergent pathways act via distinct mechanisms to enhance the accessibility of the *Igk* locus and its recombination. Restoration of IRF-4 resulted in high amounts of *Igk* germline transcription and preferentially stimulated histone H4 acetylation at the 3′Eκ enhancer. These data are compatible with molecular analyses demonstrating in vivo binding of IRF-4 to 3′Eκ via a composite Ets-IRF element (Lu et al., 2003) and a requirement for 3′Eκ in activating *Igk* germline transcription (Inlay et al., 2002). In contrast, lowering of IL-7 signaling preferentially induced histone acetylation at iEκ and robust *Rag* gene expression. Importantly, the two pathways function synergistically. The molecular basis of synergy in promoting *Igk* recombination appears to be manifested at two steps. First, each pathway targets a distinct *Igk* enhancer and synergy is likely to be a consequence of simultaneously activating both enhancers. Second, IRF-4 preferentially induces *Igk* germline transcription, whereas attenuation of IL-7 signaling more highly induces *Rag* gene expression, thereby optimizing changes in accessibility with expression of the recombinase.

Despite the fact that both pathways can promote chromatin alterations that contribute to *Igk* locus accessibility, IRF-4 specifically functions to position one *Igk* allele away from pericentromeric heterochromatin. In pre-B cells, one *Igk* allele is associated with pericentromeric heterochromatin, and this interaction has been proposed to impair V(D)J recombination and contribute to allelic exclusion (Goldmit et al., 2005). Intriguingly, *Irf4*$^{-/-}$ *Irf8*$^{-/-}$ pre-B cells have an increased number of nuclei in which both *Igk* alleles are associated with pericentromeric heterochromatin. This is in agreement with the transcriptional inactivity of the germline *Igk* loci in these cells. Interestingly, attenuation of IL-7 signaling does not alter this association pattern, whereas re-expression of IRF-4 restores the pattern observed in wild-type cells that are undergoing light-chain rearrangement. The mechanism by which IRF-4 positions an *Igk* allele away from pericentromeric heterochromatin remains to be determined. One possibility is that IRF-4 upon binding to an *Igk* allele repositions it in the nucleus by associating it with an RNA Pol II factory (Ragoczy et al., 2006), consistent with the potent activity of IRF-4 in activating *Igk* germline transcription. Alternatively, IRF-4 upon binding induces chromatin-structure alterations that are not compatible with sustaining an association with pericentromeric heterochromatin. Regardless of the mechanism, to our knowledge this is the first demonstration that a developmentally important transcriptional regulator functions to reposition endogenous target genes away from pericentromeric heterochromatin.

It has been proposed that the monoallelic activation of an *Igk* allele for transcription and recombination is limited by one or more regulatory factors that interact with the enhancers (Liang et al., 2004). We propose that IRF-4 is such a developmentally limiting determinant. Its ability to promote *Igk* germline transcription and recombination as well as repositioning of one but not both *Igk* alleles away from pericentromeric heterochromatin is consistent with this possibility. Furthermore, IRF-4 expression is upregulated in pre-B cells, and its occupancy at the 3′Eκ enhancer increases when cells transition from the pro- to the pre-B cell stage (Shaffer et al., 1997). We suggest that IRF-4 may also function as a limiting determinant in regulating *Igk* versus Igλ recombination because it promotes synergy between *Igk* but not Igλ enhancers.

Although we do not yet fully understand how IL-7 signaling inhibits *Igk* recombination, we demonstrate that IL-7 inhibits the binding of the transcription factor E2A at iEκ. E2A is required

for *Igk* recombination in pre-B cells (Lazorchak et al., 2006), and iEκ enhancer activity is critically dependent on E2A (Inlay et al., 2004). IL-7 signaling does not result in increased E2A protein expression or DNA binding activity but instead enhances its occupancy at iEκ. We note that an enhancer in the *Rag* locus also contains a functional E2A binding site (Hsu et al., 2003). Thus, the induction of *Rag1* gene transcription upon attenuation of IL-7 signaling may be also due to increased binding of E2A at the *Rag* locus. We therefore propose that IL-7 signaling functions in pre-B cells to regulate E2A accessibility at selective target genes.

IL-7 signaling positively regulates distal V_H gene histone acetylation and *Igh* recombination in pro-B cells via the transcription factor Stat5 (Bertolino et al., 2005). Paradoxically, we now demonstrate that IL-7 signaling negatively regulates histone acetylation at iEκ and *Igk* recombination. These analyses lead us to suggest that IL-7 signaling may be used to developmentally order *Igh* and *Igk* recombination events in B cell development. According to this model, in pro-B cells, IL-7 signaling would promote *Igh* recombination while inhibiting *Igk* accessibility and recombination. In pre-B cells, the attenuation of IL-7 signaling that is associated with the expression and activity of the pre-BCR would then enable efficient *Igk* recombination. Importantly, the cell-cycle arrest that is caused by attenuation of IL-7 signaling is not sufficient to induce light-chain recombination. We demonstrate that IL-7 signaling is also a potent negative regulator of *Rag* gene expression in pre-B cells. Thus, attenuation of IL-7 signaling functions to induce *Igk* locus accessibility, recombinase gene expression, and recombination of *Igk* alleles in pre-B cells. Because the pre-TCR regulates the transition from pre-T to T cell and its expression is coupled to attenuation of IL-7 signaling (Van De Wiele et al., 2004), we propose that *Rag* gene expression and the promotion of TCRα rearrangement may also be ensured by a combination of acquired pre-TCR and attenuated IL-7 signaling.

Our proposal that attenuation of IL-7 signaling contributes to the developmental ordering of immunoglobulin gene recombination is consistent with the anatomic distribution of pro-B and pre-B cells in the bone marrow. Pro-B cells are associated with IL-7-expressing stroma while pre-B cells are positioned away from such niches (Tokoyoda et al., 2004). Because the IL-7 receptor is not downregulated at the pre-B stage (Rolink et al., 2000), the positioning of pre-B cells away from IL-7-expressing stromal cells would provide a mechanism for the attenuation of IL-7 signaling. We now demonstrate that IRF-4 upregulates the expression of the chemokine receptor *Cxcr4* in pre-B cells and promotes migration toward a source of CXCL12. CXCR4 is required for early B cell development and is the sole physiological receptor for CXCL12 (Zou et al., 1998). CXCL12 is expressed on bone marrow stromal cells that are spatially separated from IL-7-expressing stromal cells (Tokoyoda et al., 2004). We suggest that IRF-4-dependent enhanced chemotaxis toward CXCL12 expressing stomal cells results in repositioning of pre-B cells from the IL-7-expressing stroma and drives their differentiation by the synergistic induction of light-chain recombination utilizing the two pathways delineated above. Intriguingly, attenuation of IL-7 signaling also upregulates *Cxcr4* expression and enhances migration toward CXCL12 (data not shown). Therefore, we suggest that altered chemotaxis of pre-B cells induced by IRF-4 would be amplified by attenuation of IL-7 signaling through a positive-feedback regulatory loop. Although, pre-B cells have increased migration toward CXCL12, they do not adhere via VCAM-1 in response to CXCL12 (Tokoyoda et al., 2004). Therefore, it is possible that migrating pre-B cells do not sustain contact with CXCL12 expressing stromal cells, consistent with the fact that they are not found to be highly associated with CXCL12 stroma in vivo.

We suggest that IRF-4,8 are a pivotal node of a regulatory circuit that drives light-chain recombination and the transition from a pre-B to B cell. Pre-B cells proliferate in response to the pre-BCR and the IL-7 receptor (IL-7R). Pre-BCR signals upregulate IRF-4, which downregulates the surrogate light-chain genes, and eventually surface pre-BCR expression (Thompson et al., 2007). Concomitantly, IRF-4 engages the 3′Eκ and the Igλ enhancers and initiates light-chain recombination. IRF-4 by upregulating *Cxcr4* causes pre-B cells to move away from IL-7-expressing stroma, thereby attenuating IL-7 signaling. This results in activation of iEκ via binding of E2A as well as optimal expression of *Rag* genes. Thus, IRF-4 coordinates pre-BCR and IL-7 signaling, thereby enabling activation of light-chain rearrangement by two distinct molecular pathways in pre-B cells.

EXPERIMENTAL PROCEDURES

Mice

The *Irf4*$^{-/-}$*Irf8*$^{-/-}$ and *Ccnd3*$^{-/-}$ mice have been previously described (Cooper et al., 2006; Lu et al., 2003). Mice were housed in specific pathogen-free conditions and were maintained and used in accordance with the Institutional Animal Care and Use Committee guidelines.

Cells and Culture Conditions

Irf4$^{-/-}$*Irf8*$^{-/-}$ bone marrow cells were isolated and positively selected for CD19 with a biotin-coupled antibody; this was followed by binding to Streptavidin microbeads (Miltenyi Biotec). Cells were cultured in Optimem media supplemented with 5% FCS and 5 ng/ml of IL-7 (IL-7^{hi}) on OP9 stromal cells. Cells were typically >99% CD19^{+}pre-BCR^{+}. IL-7 signaling was attenuated by culturing in media containing 0.1 ng/ml of IL-7 (IL-7^{lo}).

DNA Constructs

The murine IRF-4 retroviral construct has been described previously (Sciammas et al., 2006). The p27 retroviral construct was a kind gift of S. Winandy (Northwestern University).

RT-PCR

Total RNA was isolated with Trizol (Invitrogen) and cDNA was made with SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR was performed in triplicate with a SYBR green kit (Stratagene) with gene-specific primers (Table S2).

PCR Analysis of *Igk* Rearrangements

PCR with genomic DNA was performed as described (Inlay et al., 2002) (primers "a" and "b" in Figure 1; Table S2). We amplified a nonrecombined region to control for the amount of DNA (primers "a" and "c" in Figure 1; Table S2). PCR products were separated by gel electrophoresis, transferred to Hybond-N membranes (Amersham), and quantitatively analyzed on a Phosphorimager after Southern blotting. Quantitative analysis was performed by Q-PCR (primers "a" and "d" in Figure 1; Table S2). We used iEκ primers (primers "e" and "f" in Figure 1; Table S2) to control for the amount of DNA.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assays (ChIP) analysis of acetylated histones and E2A were performed essentially as previously described (Bertolino et al., 2005; Deleuze et al., 2007). ChIP PCR primers are listed in Table S2. Antibodies

used for immunoprecipitation were as follows: acetylated H4 (06-866; Upstate Biotechnology), control Ig (sc-2027; Santa Cruz), and E47 antibodies (sc-763 and sc-416; Santa Cruz).

Electrophoretic Mobility-Shift Assays

Nuclear extracts were prepared and assayed with E2A binding site oligonucleotide probes as described previously (Kee and Murre, 1998). The DNA probes used are listed in Table S2.

Retroviral Transduction of *Irf4*$^{-/-}$*Irf8*$^{-/-}$ Cells

PlatE packaging cells were transiently transfected with retroviral constructs with the Fugene reagent (Roche). Viral supernatants were collected 48 hr and 72 hr after transduction. *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells were suspended in retroviral supernatant with 8 μg/ml polybrene in the presence of IL-7 (5 ng/ml) and centrifuged at 2200 rpm for 2 hr at room temperature. Cells were incubated at 32°C for 2–4 hr and then were washed and plated on OP9 cells in the presence of 5 ng/ml of IL-7.

Immunoblotting

Protein extracts were prepared as previously described (Cooper et al., 2006). Protein lysates from equivalent cell numbers were separated by SDS-PAGE and transferred to Immobilion-P membranes (Millipore). Protein blots were probed with antibodies to cyclin D2 (M-20; Santa Cruz), cyclin D3 (C-16; Santa Cruz), p27 (sc-1641; Santa Cruz), E47 (554077; Becton Dickinson), or Stat5 (sc-835; Santa Cruz). Either actin (MAB1501; Chemicon International) or HPRT (sc-20975; Santa Cruz) was used as an internal control.

Three-Dimensional DNA FISH

Three-dimensional DNA-FISH experiments were performed as previously described (Skok et al., 2001) The *Igk* DNA probes were generated from BACs 101G13 (Vk24) and 387E13 (*Igk* constant region) in combination with a γ-satellite probe. Probes were directly labeled by nick translation with dUTP-Cy3 or dUTP-A488 (Invitrogen). The γ-satellite probe (Skok et al., 2001) was labeled with dUTP-Cy5 (GE Healthcare). Cells were analyzed by confocal microscopy on a Leica Sp5 AOBS (Acoustica Optical Beam Splitter) system. Optical sections separated by 0.3 μm were collected, and only cells with signals from both alleles (typically 95%) were analyzed.

Flow Cytometry

Surface staining and intracellular staining has been described previously (Sciammas et al., 2006). Antibodies specific for murine CD19 (1D3) and IgM (II/41) were from BD PharMingen. For cell-cycle analysis, cells were stained with 10 μg/ml Hoechst 33342 (Molecular probes) in media at 37°C for 60 min. Cells transduced cells with p27-IRES-H-2K^{k} retroviral vectors were prestained with PE anti-mouse H-2K^{k} (36-7-5; BD PharMingen). Data were collected with the FACS Calibur flow cytometer or the LSR II and were analyzed with FlowJo software (Tree Star).

Microarray Analysis

Total RNA was isolated from *Irf4*$^{-/-}$*Irf8*$^{-/-}$ cells cultured in high IL-7 (5 ng/ml) and shifted to low IL-7 (0.1 ng/ml) for 1 day. IRF-4-transduced cells were sorted and maintained in high IL-7 for 2 days before RNA isolation. Triplicates of each cell sample were used in the analysis. Biotin-labeled cRNA was hybridized to mouse Genome 430 2.0 Array according to manufacturer's instructions as described previously (Laslo et al., 2006). Expression levels of select genes were confirmed by Q-PCR.

Cell-Migration Assays

Migration assays were performed as described previously (Reif et al., 2002). A total of 0.3 to 1 × 10^{6} cells/100 ul were placed in the upper compartment of a transwell chamber (5 um pore size, Corning) with 600 ul of medium containing 100 ng/ml of CXCL12 (Sigma). The number of cells that migrated into the lower chamber was measured by flow cytometry after 2 hr and expressed relative to the number of input cells. All assays were performed in duplicate.

ACCESSION NUMBERS

All data have been deposited with the Gene Expression Omnibus at NCBI under the accession number GSE10273.

SUPPLEMENTAL DATA

Eight figures and two tables are available at http://www.immunity.com/cgi/content/full/28/3/335/DC1/.

ACKNOWLEDGMENTS

We thank P. Sicinski for use of *Ccnd3*$^{-/-}$ mice. K.J. is supported by a fellowship from the Leukemia Lymphoma Society. T.H. is supported by a fellowship from The International Human Frontier Science Program Organization. J.A.S. is supported by the Wellcome Trust and NYU start-up funds. I.A. is supported by NIH grant R01CA105129. H.S. is an Investigator with the Howard Hughes Medical Institute.

Received: September 27, 2007
Revised: December 4, 2007
Accepted: December 20, 2007
Published online: February 14, 2008

REFERENCES

Bertolino, E., Reddy, K., Medina, K.L., Parganas, E., Ihle, J., and Singh, H. (2005). Regulation of interleukin 7-dependent immunoglobulin heavy-chain variable gene rearrangements by transcription factor STAT5. Nat. Immunol. *6*, 836–843.

Cooper, A.B., Sawai, C.M., Sicinska, E., Powers, S.E., Sicinski, P., Clark, M.R., and Aifantis, I. (2006). A unique function for cyclin D3 in early B cell development. Nat. Immunol. *7*, 489–497.

Deleuze, V., Chalhoub, E., El-Hajj, R., Dohet, C., Le Clech, M., Couraud, P.O., Huber, P., and Mathieu, D. (2007). TAL-1/SCL and its partners E47 and LMO2 up-regulate VE-cadherin expression in endothelial cells. Mol. Cell. Biol. *27*, 2687–2697.

Eisenbeis, C.F., Singh, H., and Storb, U. (1995). Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator. Genes Dev. *9*, 1377–1387.

Flemming, A., Brummer, T., Reth, M., and Jumaa, H. (2003). The adaptor protein SLP-65 acts as a tumor suppressor that limits pre-B cell expansion. Nat. Immunol. *4*, 38–43.

Geier, J.K., and Schlissel, M.S. (2006). Pre-BCR signals and the control of Ig gene rearrangements. Semin. Immunol. *18*, 31–39.

Goldmit, M., Ji, Y., Skok, J., Roldan, E., Jung, S., Cedar, H., and Bergman, Y. (2005). Epigenetic ontogeny of the Igk locus during B cell development. Nat. Immunol. *6*, 198–203.

Grawunder, U., Haasner, D., Melchers, F., and Rolink, A. (1993). Rearrangement and expression of kappa light chain genes can occur without mu heavy chain expression during differentiation of pre-B cells. Int. Immunol. *5*, 1609–1618.

Hsu, L.Y., Lauring, J., Liang, H.E., Greenbaum, S., Cado, D., Zhuang, Y., and Schlissel, M.S. (2003). A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. Immunity *19*, 105–117.

Inlay, M., Alt, F.W., Baltimore, D., and Xu, Y. (2002). Essential roles of the kappa light chain intronic enhancer and 3′ enhancer in kappa rearrangement and demethylation. Nat. Immunol. *3*, 463–468.

Inlay, M.A., Tian, H., Lin, T., and Xu, Y. (2004). Important roles for E protein binding sites within the immunoglobulin kappa chain intronic enhancer in activating Vkappa Jkappa rearrangement. J. Exp. Med. *200*, 1205–1211.

Kee, B.L., and Murre, C. (1998). Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. J. Exp. Med. *188*, 699–713.

Klein, U., Casola, S., Cattoretti, G., Shen, Q., Lia, M., Mo, T., Ludwig, T., Rajewsky, K., and Dalla-Favera, R. (2006). Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. Nat. Immunol. *7*, 773–782.

Kosak, S.T., Skok, J.A., Medina, K.L., Riblet, R., Le Beau, M.M., Fisher, A.G., and Singh, H. (2002). Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. Science *296*, 158–162.

Laslo, P., Spooner, C.J., Warmflash, A., Lancki, D.W., Lee, H.J., Sciammas, R., Gantner, B.N., Dinner, A.R., and Singh, H. (2006). Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. Cell *126*, 755–766.

Lazorchak, A.S., Schlissel, M.S., and Zhuang, Y. (2006). E2A and IRF-4/Pip promote chromatin modification and transcription of the immunoglobulin kappa locus in pre-B cells. Mol. Cell. Biol. *26*, 810–821.

Liang, H.E., Hsu, L.Y., Cado, D., and Schlissel, M.S. (2004). Variegated transcriptional activation of the immunoglobulin kappa locus in pre-b cells contributes to the allelic exclusion of light-chain expression. Cell *118*, 19–29.

Lu, R., Medina, K.L., Lancki, D.W., and Singh, H. (2003). IRF-4,8 orchestrate the pre-B-to-B transition in lymphocyte development. Genes Dev. *17*, 1703–1708.

Ma, S., Turetsky, A., Trinh, L., and Lu, R. (2006). IFN regulatory factor 4 and 8 promote Ig light chain kappa locus activation in pre-B cell development. J. Immunol. *177*, 7898–7904.

Matsuyama, T., Grossman, A., Mittrucker, H.W., Siderovski, D.P., Kiefer, F., Kawakami, T., Richardson, C.D., Taniguchi, T., Yoshinaga, S.K., and Mak, T.W. (1995). Molecular cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE). Nucleic Acids Res. *23*, 2127–2136.

Milne, C.D., Fleming, H.E., and Paige, C.J. (2004). IL-7 does not prevent pro-B/pre-B cell maturation to the immature/sIgM(+) stage. Eur. J. Immunol. *34*, 2647–2655.

Milne, C.D., and Paige, C.J. (2006). IL-7: A key regulator of B lymphopoiesis. Semin. Immunol. *18*, 20–30.

Muljo, S.A., and Schlissel, M.S. (2003). A small molecule Abl kinase inhibitor induces differentiation of Abelson virus-transformed pre-B cell lines. Nat. Immunol. *4*, 31–37.

Pongubala, J.M., Nagulapalli, S., Klemsz, M.J., McKercher, S.R., Maki, R.A., and Atchison, M.L. (1992). PU.1 recruits a second nuclear factor to a site important for immunoglobulin kappa 3′ enhancer activity. Mol. Cell. Biol. *12*, 368–378.

Quong, M.W., Martensson, A., Langerak, A.W., Rivera, R.R., Nemazee, D., and Murre, C. (2004). Receptor editing and marginal zone B cell development are regulated by the helix-loop-helix protein, E2A. J. Exp. Med. *199*, 1101–1112.

Ragoczy, T., Bender, M.A., Telling, A., Byron, R., and Groudine, M. (2006). The locus control region is required for association of the murine beta-globin locus with engaged transcription factories during erythroid maturation. Genes Dev. *20*, 1447–1457.

Reif, K., Ekland, E.H., Ohl, L., Nakano, H., Lipp, M., Forster, R., and Cyster, J.G. (2002). Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. Nature *416*, 94–99.

Rolink, A.G., Winkler, T., Melchers, F., and Andersson, J. (2000). Precursor B cell receptor-dependent B cell proliferation and differentiation does not require the bone marrow or fetal liver environment. J. Exp. Med. *191*, 23–32.

Sato, H., Saito-Ohara, F., Inazawa, J., and Kudo, A. (2004). Pax-5 is essential for kappa sterile transcription during Ig kappa chain gene rearrangement. J. Immunol. *172*, 4858–4865.

Schlissel, M.S. (2004). Regulation of activation and recombination of the murine Igkappa locus. Immunol. Rev. *200*, 215–223.

Sciammas, R., Shaffer, A.L., Schatz, J.H., Zhao, H., Staudt, L.M., and Singh, H. (2006). Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. Immunity *25*, 225–236.

Shaffer, A.L., Peng, A., and Schlissel, M.S. (1997). In vivo occupancy of the kappa light chain enhancers in primary pro- and pre-B cells: A model for kappa locus activation. Immunity *6*, 131–143.

Shaw, A.C., Swat, W., Davidson, L., and Alt, F.W. (1999). Induction of Ig light chain gene rearrangement in heavy chain-deficient B cells by activated Ras. Proc. Natl. Acad. Sci. USA *96*, 2239–2243.

Skok, J.A., Brown, K.E., Azuara, V., Caparros, M.-L., Baxter, J., Takacs, K., Dillon, N., Gray, D., Perry, R.P., Merkenschlager, M., and Fisher, A.G. (2001). Nonequivalent nuclear location of immunoglobulin alleles in B lymphocytes. Nat. Immunol. *2*, 848–854.

Stanhope-Baker, P., Hudson, K.M., Shaffer, A.L., Constantinescu, A., and Schlissel, M.S. (1996). Cell type-specific chromatin structure determines the targeting of V(D)J recombinase activity in vitro. Cell *85*, 887–897.

Thompson, E.C., Cobb, B.S., Sabbattini, P., Meixlsperger, S., Parelho, V., Liberg, D., Taylor, B., Dillon, N., Georgopoulos, K., Jumaa, H., et al. (2007). Ikaros DNA-binding proteins as integral components of B cell developmental-stage-specific regulatory circuits. Immunity *26*, 335–344.

Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B.I., and Nagasawa, T. (2004). Cellular niches controlling B lymphocyte behavior within bone marrow during development. Immunity *20*, 707–718.

Van De Wiele, C.J., Marino, J.H., Murray, B.W., Vo, S.S., Whetsell, M.E., and Teague, T.K. (2004). Thymocytes between the beta-selection and positive selection checkpoints are nonresponsive to IL-7 as assessed by STAT-5 phosphorylation. J. Immunol. *172*, 4235–4244.

Xu, S., Lee, K.G., Huo, J., Kurosaki, T., and Lam, K.P. (2007). Combined deficiencies in Bruton tyrosine kinase and phospholipase Cgamma2 arrest B-cell development at a pre-BCR+ stage. Blood *109*, 3377–3384.

Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., and Littman, D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature *393*, 595–599.